# Characterization of Gate 2- protocol

## Materials and Equipment:

- *E. coli*:
  - K-12 Top 10 containing two plasmids: pSB1C3-Plac-LuxR and pSB1A2-Plux-GFP -> **Amp+CM resistance**!
  - Top10 containing the plasmid pSB1C3 (blank) -> **CM resistance!**
- 150ml LB (100ml per the strain above + 50ml for the blank)
- 150ml BA (100ml per the strain above + 50ml for the blank)
- 50ml Falcons
- Antibiotics (CM + AMP)
- 1 Erlenmeyer flask 500ml
- 1 Erlenmeyer flask 250ml
- Measuring glass 100ml
- 1x 48 well plate **– label A-F and 1-8**
- 3o6c-AHL from Sigma-Aldrich [140mM]
- IPTG [1M]
- 96 microwells for plate reader
- Plate reader

## Protocol:

1. Prepare a starter by growing the cells in LB medium + appropriate antibiotics at 37°C overnight (in a 50ml falcon).
2. Transfer 1/0.5ml starter to 100/50ml LB containing appropriate antibiotics (in 500ml/250ml Erlenmeyer flask).
3. Grow at 37°C until $OD_{600}$ of 0.6 (Check the O.D after 90 minutes).
   - For the spectrofotometer OD test use 1ml of LB medium as blank and then measure the OD by using 1ml from the sample.
4. Transfer the samples to 50ml Falcon tubes and mark the volume on the falcon itself. If the Erlenmeyer contains more than 50ml transfer the remaining culture to another falcon tube.
5. Pellet cells (centrifuge for 10 min at 4000rpm) and then pour off the supernatant.
6. Vortex to break the pellet.

7. Add bioassay medium (BA) to the falcons up to the volume marked. Add appropriate antibiotics (needs to be diluted 1:1000). Write on the falcons the following names:
   - pSB1C3-Plac-LuxR + pSB1A2-Plux-GFP, Amp+ CM+, (**A**)
   - pSB1C3, CM+, (**B**)
8. Transfer 7ml from **falcon A** to a new falcon (**C**). Add to **falcon C** 5µl AHL [140mM] to get a solution with AHL concentration of 100µM. Add to **falcon C** 7µl IPTG 1M to get a solution with IPTG concentration of 1mM. Re-suspend the cells in this medium by vortex.
   - Transfer 2ml from **falcon C** to wells A1 and A2 in your 48 wells plate. **2ml in each well!!**
   - Transfer 1ml from **falcon C** to wells A3 and A4 in your 48 wells plate. **1ml in each well!!**
9. Transfer 45ml from **falcon A** to a new falcon (**D**). Add to **falcon D** 45µl IPTG 1M to get a solution with IPTG concentration of 1mM. Re-suspend the cells in this medium by vortex.
10. To get an AHL concentration of 50µM, transfer to wells A3 and A4 1 ml from **falcon D.**
11. Add to each well from column B to column E (only rows 1-4) and wells F1, F2, F3, F4 1.8ml from **falcon D**.
12. For the blank: Transfer 5ml from **falcon B** to a new falcon (**E**). Add to **falcon E** 3.6µl AHL [140mM] to get a solution with AHL concentration of 100µM. Add to **falcon E** 5µl IPTG 1M to get a solution with IPTG concentration of 1mM. Re-suspend the cells in this medium by vortex.
    - Transfer 1.8ml from **falcon E** to wells C5 and C6 in your 48 wells plate.
    - Transfer 4ml from **falcon B** to a new falcon (**F**) and add 4µl of IPTG 1M to get a solution with IPTG concentration of 1mM. Transfer 1.8ml from **falcon F** to wells D5 and D6 in your 48 wells plate.

13. For the negative control (without IPTG): Transfer 5ml from **falcon A** to a new falcon (**G**). Add to **falcon G** 3.6µl AHL [140mM] to get a solution with AHL concentration of 100µM.
    - Transfer 1.8ml from **falcon G** to wells A5 and A6 in your 48 wells plate.
    - Transfer 1.8ml from **falcon A** to wells B5 and B6 in your 48 wells plate.
14. Perform x10 dilutions as follows (use the multipipettor with the program pipette and mix) :
    **Only rows 1-4**: transfer 200µl from wells in column A to column B. Transfer 200µl from wells in column B to column C. **Continue in this fashion until column E**. Dispose 200µl **from column E in rows 3 and 4 only!** For rows 1 and 2, transfer 200µl from wells in column E to column F. Dispose 200µl **from column F in rows 1 and 2 only!**
15. Incubate the plate in shaker for 3 hours in 37°C.
16. After 3 hours remove 200µl from each well and transfer to the plate-reader micro-well. Read absorbance ($OD_{600}$ – for cell concentration) and fluorescence (excitation peak: 501nm, emission peak: 511nm) with plate reader. Repeat every 0.5 hour for 2 hours. Use each 96 microwell plate for two readings. Use half for one reading and the other half for another reading. (see table 2).
17. Save tables of the results.
18. Calculate the relative expression:

$$Expression = \frac{F}{OD_{600}} - \left(\frac{F}{OD_{600}}\right)_{blank}$$

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | AHL 100µM (+IPTG, +AHL) | AHL 10µM (+IPTG, +AHL) | AHL 1µM (+IPTG, +AHL) | AHL 100nM (+IPTG, +AHL) | AHL 10nM (+IPTG, +AHL) | AHL 1nM (+IPTG, +AHL) |
| 2 | AHL 100µM (+IPTG, +AHL) | AHL 10µM (+IPTG, +AHL) | AHL 1µM (+IPTG, +AHL) | AHL 100nM (+IPTG, +AHL) | AHL 10nM (+IPTG, +AHL) | AHL 1nM (+IPTG, +AHL) |
| 3 | AHL 50µM (+IPTG, +AHL) | AHL 5µM (+IPTG, +AHL) | AHL 5µM (+IPTG, +AHL) | AHL 50nM (+IPTG, +AHL) | AHL 5nM (+IPTG, +AHL) | AHL 0nM (+IPTG) |
| 4 | AHL 50µM (+IPTG, +AHL) | AHL 5µM (+IPTG, +AHL) | AHL 5µM (+IPTG, +AHL) | AHL 50nM (+IPTG, +AHL) | AHL 5nM (+IPTG, +AHL) | AHL 0nM (+IPTG) |
| 5 | **AHL 100µM (+AHL)** | **AHL 0µM** | **AHL 100µM (+IPTG, +AHL)** | **AHL 0nM (+IPTG)** | | |
| 6 | **AHL 100µM (+AHL)** | **AHL 0µM** | **AHL 100µM (+IPTG, +AHL)** | **AHL 0nM (+IPTG)** | | |
| 7 | | | | | | |
| 8 | | | | | | |

Table 1: 48 well plate reader

Black: Culture of TOP10 with two plasmids (pSB1C3-Plac-LuxR and pSB1A2-Plux-GFP), IPTG, CM and Amp.

**Red: Blank- culture of TOP10 with pSB1C3.**

Blue: Culture of TOP10 with two plasmids (pSB1C3-Plac-LuxR and pSB1A2-Plux-GFP), CM and Amp (without IPTG).